

CUL1 regulates TOC1 protein stability in the Arabidopsis circadian clock

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Summary

The circadian clock is the endogenous timer that coordinates physiological processes with daily and seasonal environmental changes. In *Arabidopsis thaliana*, establishment of the circadian period relies on targeted degradation of TIMING OF CAB EXPRESSION 1 (TOC1) by the 26S proteasome. ZEITLUPE (ZTL) is the F-box protein that associates with the SCF (Skp/Cullin/F-box) E3 ubiquitin ligase that is responsible for marking TOC1 for turnover. CULLIN1 (CUL1) is a core component of SCF complexes and is involved in multiple signaling pathways. To assess the contribution of CUL1-containing SCF complexes to signaling within the plant oscillator, circadian rhythms were examined in the recessive, temperature-sensitive *CUL1* allele *axr6-3*. The activity of CUL1 in this mutant declines progressively with increasing ambient temperature, resulting in more severe defects in CUL1-dependent activities at elevated temperature. Examination of circadian rhythms in *axr6-3* revealed circadian phenotypes comparable to those observed in *ztl* null mutants; namely, lengthened circadian period, altered expression of core oscillator genes, and limited degradation of TOC1. In addition, treatment of seedlings with exogenous auxin did not alter TOC1 stability. These results demonstrate that CUL1 is required for TOC1 degradation and further suggest that this protein is the functional cullin for the SCF^{ZTL} complex.

Keywords: circadian rhythms, CUL1, post-translational regulation, ubiquitin ligase, proteasome, *Arabidopsis*.

Introduction

The circadian oscillator is the endogenous timekeeper responsible for generating and maintaining physiological rhythms with a period of approximately 24 h. Substantial progress has been made in understanding the molecular mechanism underlying the plant clock and the consequences of its rhythms for plant physiology (Harmer *et al.*, 2005; Harmon *et al.*, 2005). Beyond providing a simple list of components for the oscillator, circadian research in *Arabidopsis thaliana* has established that the clock serves to enhance plant fitness by influencing myriad physiological processes (Hotta *et al.*, 2007). The circadian clock is perhaps best known for its contribution to the measurement of day length, which triggers flowering during inductive photoperiods (Imaizumi and Kay, 2006). The clock also participates in responses to additional external stimuli, including

neighbor shading, low temperature, and other abiotic stresses (Fowler *et al.*, 2005; Kreps *et al.*, 2002; Salter *et al.*, 2003). In addition, circadian regulation has been observed for many aspects of metabolism, including starch production/utilization, transpiration, and nitrate assimilation (Blasing *et al.*, 2005; Kreps and Kay, 1997). The plant growth rate is also closely tied to circadian clock function (Dodd *et al.*, 2005; Green *et al.*, 2002).

The ubiquitin/26S proteasome pathway is a key means of controlling protein activity in plants, fungi, and animals (Hershko and Ciechanover, 1998; Smalle and Vierstra, 2004). The central regulatory mechanism of this pathway is selective degradation of proteins by ubiquitinylation, which involves covalent attachment of the small protein ubiquitin to a specific target protein. Ubiquitin serves as a signal for

target protein degradation, mediated by the 26S proteasome (Pickart, 2001). Plants have significantly expanded the use of this pathway to control the activity and expression of proteins: genes encoding components of the ubiquitin/26S proteasome comprise approximately 5% of the Arabidopsis genome (Bachmair *et al.*, 2001; Gagne *et al.*, 2002).

Ubiquitylation takes place at the end of a multi-step process and is mediated by specific E3 ubiquitin ligases that are responsible for identifying cognate target proteins and for final transfer of ubiquitin to the target protein (Pickart, 2001). Four categories of E3 ubiquitin ligases have been confirmed in plants, the most abundant being the SCF (for SKP/CULLIN/F-box) complex (Vierstra, 2003). As its name indicates, the SCF complex is a multi-protein assembly composed of individual CULLIN (CUL), SKP (ASK), and F-box subunits, in addition to one member of a class of RING finger proteins known as RBX. The function of the F-box protein is to identify the target protein; therefore, the F-box protein provides target specificity to the E3 ligase. Among the SCF subunits in plants, F-box proteins exhibit the greatest diversity, as over 700 of these proteins are encoded in the Arabidopsis genome (Gagne *et al.*, 2002). CULs are the core subunits that act as scaffolds for assembly of the complex (Zheng *et al.*, 2002). Of the eleven CUL genes predicted in Arabidopsis, only five (CUL1, CUL2, CUL3A, CUL3B, and CUL4) encode canonical CUL proteins (Risseuw *et al.*, 2003). The activity of CUL1 is required for embryogenesis, senescence, floral development and responses to phytohormones and light (Durfee *et al.*, 2003; Gray *et al.*, 2002; Guo and Ecker, 2003; Hellmann *et al.*, 2003; Potuschak *et al.*, 2003; Shen *et al.*, 2002; Woo *et al.*, 2001). Homozygous null alleles of CUL1 confer an embryo-lethal phenotype, suggesting an essential role for SCF ubiquitin ligases early in plant development (Shen *et al.*, 2002). Although CUL1 was thought to participate in the circadian clock (Han *et al.*, 2004), there is no functional evidence confirming its role in the oscillator.

axr6-3 is a temperature-sensitive *CUL1* mutant that is recessive and fully viable (Quint *et al.*, 2005). This mutant has defects in flower development and in responses to the phytohormones auxin and jasmonate. The severity of the *axr6-3* phenotype is progressively stronger with increasing ambient temperature; for example, the concentrations of auxin required to inhibit root growth by 50% in *axr6-3* at 24 and 28°C are 10- and 100-fold greater, respectively, than that needed at 20°C (Quint *et al.*, 2005). *axr6-3* harbors a missense mutation in CUL1, substituting a glutamic acid at position 159 for a lysine, which renders the protein unstable at elevated temperatures and limits the formation of active SCF complexes harboring CUL1 (Quint *et al.*, 2005). The temperature-sensitive and viable nature of *axr6-3* makes it a unique tool to investigate the contribution of CUL1 to plant signaling pathways.

The ubiquitin/26S proteasome pathway plays an important role in the establishment of period length in the Arabidopsis circadian system (Kim *et al.*, 2007; Mas *et al.*, 2003b). ZEITLUPE (ZTL) is an F-box/LOV/Kelch protein (Han *et al.*, 2004; Somers *et al.*, 2000) that acts as a circadian clock-specific blue-light photoreceptor (Kim *et al.*, 2007). TIMING OF CAB EXPRESSION 1 (TOC1) is a pseudo-response regulator protein that is presumed to function as a transcription factor (Strayer *et al.*, 2000). TOC1 lengthens the circadian period (Makino *et al.*, 2002; Mas *et al.*, 2003a), and ZTL acts antagonistically to shorten period (Somers *et al.*, 2004). Strong constitutive overexpression of *TOC1* drives the clock to arrhythmicity (Makino *et al.*, 2002; Mas *et al.*, 2003a). *ztl* loss-of-function mutants exhibit long periods (Jarillo *et al.*, 2001; Somers *et al.*, 2000), whereas *toc1* mutants have short periods (Mas *et al.*, 2003a; Somers *et al.*, 1998; Strayer *et al.*, 2000). The *ztl toc1* double mutant also has a short period (Mas *et al.*, 2003b), indicating that the *ztl* phenotype requires TOC1 function. TOC1 and ZTL physically interact *in vivo*, and TOC1 accumulation is constitutively high in a *ztl* background (Mas *et al.*, 2003b); hence, the F-box protein ZTL targets TOC1 for proteasome-mediated degradation. ZTL is critical for controlling TOC1 activity, which, in turn, is important for modulation of the length of the circadian period.

Although ZTL is associated with CUL1 *in vivo* (Han *et al.*, 2004), CUL1 has not been directly demonstrated to be functionally important for SCF^{ZTL} activity. Furthermore, it remains unclear whether SCF complexes harboring CUL1 or other CULs contribute at any point to the generation and maintenance of circadian rhythms. To determine the role that SCF complexes reliant on CUL1 play in the Arabidopsis molecular clock, we examined circadian rhythms in *axr6-3*. We found that *axr6-3* plants exhibited strong circadian phenotypes, which correlated with the loss of SCF^{ZTL} activity associated with *ztl* null alleles, including a substantial phase delay for circadian gene expression, altered expression levels for core clock genes, and a long circadian period. Circadian rhythms in the CUL1 mutant were also less precise; the period varied substantially within a group of seedlings, such that a larger proportion of the individuals were scored as arrhythmic. Furthermore, inactivation of CUL1 by elevated temperature blocked turnover of TOC1. These findings indicate that CUL1 activity is required for TOC1 degradation, consistent with CUL1 acting as the primary CUL protein in SCF^{ZTL}.

Results

Leaf movement rhythms in axr6-3 are temperature-dependent

To assess the impact of diminished CUL1 activity on circadian rhythms, *axr6-3* and wild-type (WT) seedlings were

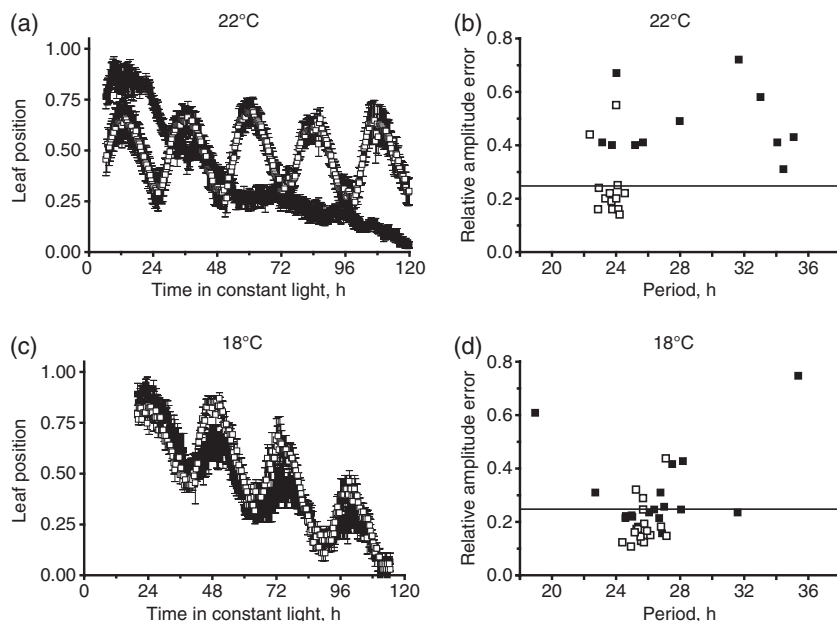


Figure 1. Rhythmic leaf movement in *axr6-3* is temperature-dependent.

(a, c) Traces of leaf position for seedlings of *axr6-3* (filled squares), and WT (open squares) in constant light at 22°C (a) and 18°C (c). Mean values ($n = 6$) are shown for each time point, and error bars indicate SEM. Data in (a) and (c) are from one of two independent experiments. The leaf position under each condition was normalized to the highest value in that experiment.

(b, d) Relative amplitude error versus estimated free-running period for *axr6-3* (filled squares) and WT (open squares) seedlings as determined by FFT/NLLS analysis at 22°C (b) and 18°C (d). Relative amplitude error values ≤ 0.25 were considered rhythmic, and this cut-off is indicated by the horizontal line. Data in (b) and (d) are from two independent experiments.

assayed for leaf movement rhythms at 22°C, which is a restrictive temperature for *axr6-3*. Seedlings were grown in 12 h light:12 h dark cycles for 5 days, and then transferred at Zeitgeber Time 0 (ZT0; ZT is expressed in hours, and ZT0 represents 08:00 h) to constant light conditions. Seedlings were assayed for rhythmic leaf movements for 5 days under these free-running conditions. *axr6-3* seedlings were arrhythmic for leaf movement when assayed at 22°C (Figure 1a). The average leaf movement trace for mutant seedlings clearly shows that these seedlings could not sustain rhythms after the first day in constant light. Conversely, WT seedlings exhibited obvious leaf movement rhythms that persisted over the entire 5 days of the experiment (Figure 1a). *axr6-3* appeared capable of entrainment, however, as a peak of the same approximate phase as in WT was apparent during the first day in constant light.

Curve-fitting analysis of the leaf movement traces confirmed that rhythmic leaf movement for all *axr6-3* seedlings was compromised under these conditions (Table 1 and Figure 1b). The estimated period length for *axr6-3* individuals was widely distributed across a broad spectrum of estimates, and a greater proportion of the seedlings were defined as arrhythmic, consistent with a lack of robust circadian behavior in *axr6-3* (Figure 1b). On the other hand, all the WT seedlings, with the exception of two, were rhythmic under the same conditions, and the period estimates tightly clustered around a value of 23.8 ± 0.5 h (Table 1 and Figure 1b). These results suggest that *axr6-3* lacks circadian leaf movements at this restrictive temperature, and therefore that CUL1 activity is important for maintenance of circadian rhythms in constant light.

To verify that the arrhythmic phenotype in *axr6-3* was due to temperature-dependent inactivation of CUL1 activity,

Table 1 Circadian leaf movement in WT and *axr6-3* seedlings at 22°C and 18°C

Genotype	22°C ^a		18°C ^a	
	Period \pm SD ^b (h)	Rhythmic/ total	Period \pm SD ^b (h)	Rhythmic/ total
WT	23.8 ± 0.5	11/13	25.7 ± 0.7	14/17
<i>axr6-3</i>	ARR ^c	0/12	26.0 ± 1.1	11/18

Plants were entrained for 5 days under 12 h light:12 h dark cycles at the indicated temperature, then held in continuous white light ($50 \mu\text{mol m}^{-2} \text{sec}^{-1}$) for 5 days.

^aData from two independent replicates.

^bThe variance-weighted mean period was determined as described previously (Millar *et al.*, 1995b; Plautz *et al.*, 1997). Only those period estimates with relative amplitude error values ≤ 0.25 and periods between 20 and 30 h were included in this analysis. SD, standard deviation.

^cARR, arrhythmic (indicates the absence of period estimates with relative amplitude error values ≤ 0.25 for any of the seedlings).

rhythms in cotyledon movement were examined in seedlings exposed to 18°C (Figure 1c,d). Previous work demonstrated that growth of *axr6-3* at 20°C enhances the auxin response in mutant seedlings, but does not restore full CUL1 activity (Quint *et al.*, 2005). We found that, at 18°C, cotyledon movement in *axr6-3* was more rhythmic than at 22°C, and the rhythms were superficially comparable to those of WT (Figure 1c). Curve-fit analysis demonstrated that the average period of the oscillator in rhythmic *axr6-3* seedlings was 26.0 ± 1.1 h, which is similar to the 25.7 ± 0.7 h period observed for WT (Table 1). However, *axr6-3* individuals exhibited a larger range of rhythmic period estimates than did WT (Figure 1d), and more *axr6-3* seedlings were arrhythmic than WT (Table 1); thus, this less restrictive temperature

only partially suppressed the *axr6-3* circadian defect in leaf movement. Clearly, robust leaf movement rhythms require full CUL1 activity, which suggests an important role in the circadian oscillator for those SCF E3 ubiquitin ligases that require CUL1 activity.

axr6-3 affects circadian period and maintenance of rhythms

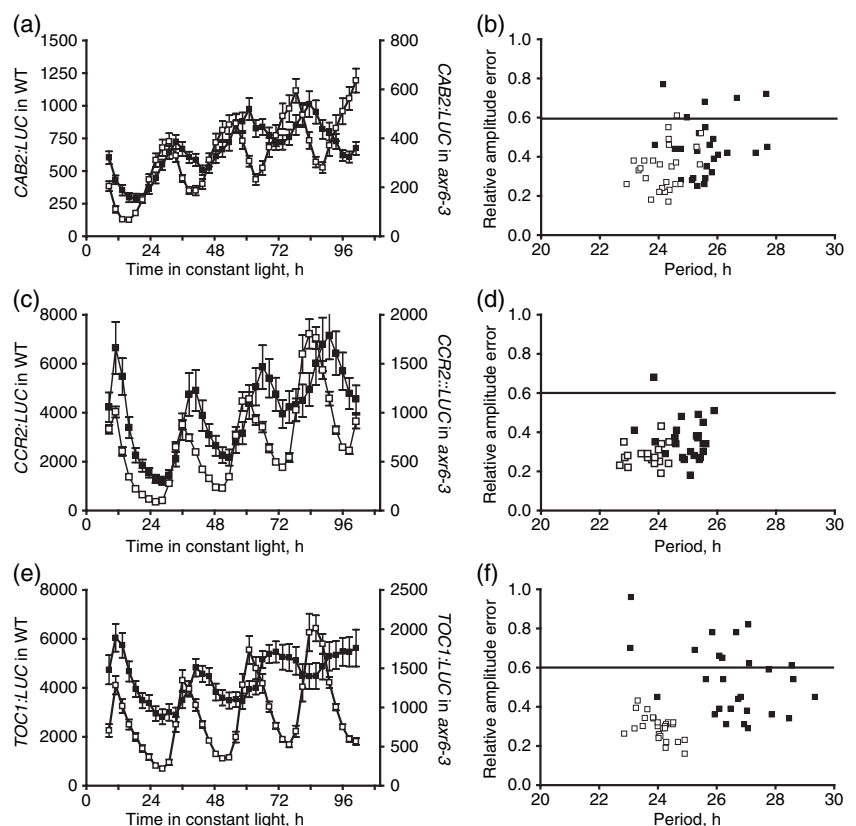
Leaf movement is an indirect measure of oscillator function, and the physiological changes driving leaf movement could have relied on CUL1-dependent processes separate from the circadian clock. Therefore, the leaf movement phenotype in *axr6-3* may have represented an amalgam of phenotypes to which the oscillator was one of several contributors. To more directly assess the state of the circadian clock in *axr6-3*, circadian gene expression was examined in the WT and mutant backgrounds by well-characterized circadian promoter:luciferase (LUC) reporter constructs. The *axr6-3* allele was crossed into plants harboring DNA constructs of *CHLOROPHYLL a/b-BINDING PROTEIN 2* (*CAB2*):LUC, *COLD CIRCADIAN RHYTHM-RNA BINDING 2* (*CCR2*):LUC, and *TOC1*:LUC, and these lines were examined for rhythmic gene expression under constant light conditions after entrainment under 12 h light:12 h dark cycles at 22°C for 7 days. The bioluminescence signal from *axr6-3* seedlings was consistently lower than from WT for each of the reporter

constructs (Figure 2a,c,e). The lower bioluminescence signal from *axr6-3* seedlings appeared to be due to the small size of the *axr6-3* seedlings rather than changes in the amplitude of gene expression, as the area of the cotyledons from mutant seedlings was consistently smaller than in WT seedlings (data not shown), and transcript levels for *CAB2*, *CCR2*, and *TOC1* in *axr6-3* were comparable to WT levels when examined by real-time PCR (Figure 3c and Figure S1a,b).

Clock-driven gene expression in *axr6-3* clearly deviated from that in WT with respect to both period length and precision of the rhythms, regardless of the reporter construct assayed (Figure 2). Visual inspection of the curves revealed a consistent phase delay in the traces from *axr6-3* seedlings, which ranged from 2.5 to 5 h, depending upon the reporter (Figure 2a,c,e). *TOC1*:LUC expression also exhibited an obvious lengthening of period, such that, under constant light conditions, expression in *axr6-3* was anti-phase to WT by the fourth day (Figure 2e). A comparable period change was not apparent in the curves for either *CAB2*:LUC or *CCR2*:LUC (Figure 2a,c). The estimated period of expression for each reporter was determined, and these results indicated that the pace of the clock in those *axr6-3* seedlings exhibiting detectable rhythms was slower than in WT (Table 2). In *axr6-3*, the average period for expression of the two reporters representing the circadian output genes

Figure 2. Circadian gene expression in *axr6-3* is long-period and rhythms lack the precision of those in WT.

(a, c, e) Bioluminescence rhythms for *CAB2*:LUC (a), *CCR2*:LUC (c), and *TOC1*:LUC (e) in *axr6-3* (filled squares) and WT (open squares) seedlings at 22°C. The left y-axis shows the bioluminescence signal for WT and the right y-axis shows that for *axr6-3*. Data are mean values ($n = 15$ for *axr6-3* and $n = 10$ for WT), and error bars indicate SEM. Representative data in (a), (c), and (e) are from one of two independent experiments. (b, d, f) Relative amplitude error versus estimated free-running period for *CAB2*:LUC (b), *CCR2*:LUC (d), and *TOC1*:LUC (f) for *axr6-3* (filled squares) and WT (open squares) seedlings as determined by FFT/NLLS analysis. Relative amplitude error values ≤ 0.6 were considered rhythmic, and this cut-off is indicated by the horizontal line. Data in (b), (d), and (f) are from two independent experiments.



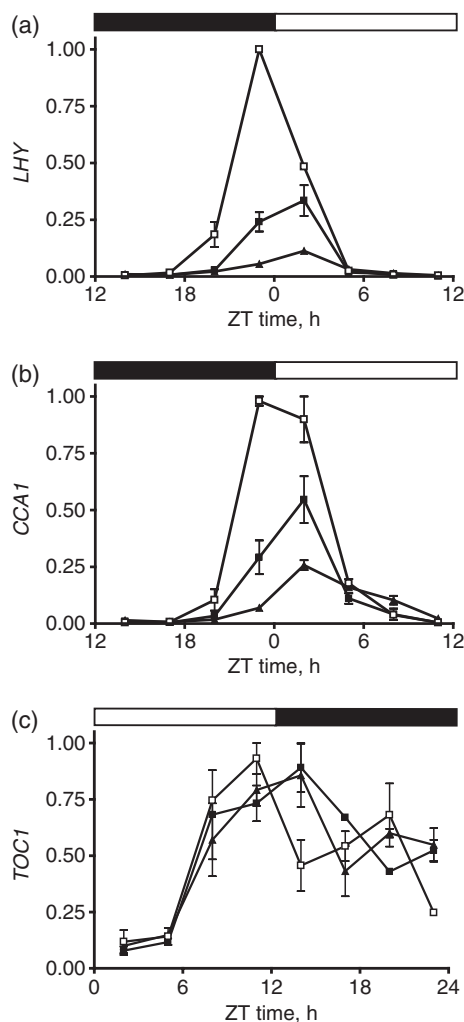


Figure 3. Clock gene expression in *axr6-3* is comparable to that of *ztl-4*. *LHY* (a), *CCA1* (b), and *TOC1* (c) transcript abundance in 1-week-old seedlings of WT (open squares), *axr6-3* (filled squares), and *ztl-4* (filled triangles), each grown under diurnal conditions. The black and white bars above the graphs indicate periods of dark and light, respectively. Data are the means of two independent replicates and error bars indicate the range. Gene expression was determined by real-time PCR, and expression of *IPP2* (see Experimental procedures) was used as a normalization control. The expression level for each experimental gene was calculated using the formula $2^{[C_T(IPP2) - C_T(\text{experimental})]}$, where C_T is the average threshold cycle for three technical replicates. The expression value for each gene was normalized to the highest value for each experiment.

CAB2:LUC, and *CCR2:LUC* was longer by about 1.5 h (Table 2). As inferred from visual inspection, the strongest period phenotype was observed for *TOC1:LUC* expression, where the average period for rhythmic *axr6-3* seedlings was 3 h longer than WT and 1.5 h longer than expression of the other two reporters (Table 2). The strong effect of *axr6-3* on *TOC1* expression suggests that CUL1 may participate directly in the regulation of this gene, whereas the changes observed for *CCR2* and *CAB2* expression may reflect contributions of CUL1 to overall clock function.

The impact of *axr6-3* on circadian gene expression was more apparent when the rhythms for individual seedlings were compared within each genotype. In general, period length in *axr6-3* seedlings was less uniform than in WT, and a greater proportion of seedlings exhibited poor rhythms, with relative amplitude error values ≤ 0.6 (Figure 2b,d,g and Table 2). For *CAB2:LUC*, the period length was spread over a broad range of values that overlapped with those for WT, and more mutant individuals were scored as arrhythmic (20% in *axr6-3* versus 5% in WT; Figure 2b and Table 2). In contrast, the period of *CCR2:LUC* expression in most of the *axr6-3* seedlings clustered around a common period value that was 1.5 h longer than in WT (Figure 2f). Furthermore, only one *axr6-3* seedling was considered arrhythmic for *CCR2:LUC* expression (Table 2). The period of *TOC1:LUC* expression was consistently 3 h longer than WT (Table 2), but the spread of these longer period values was much greater than in WT (Figure 2d). In addition, 30% of *axr6-3* individuals showed arrhythmic *TOC1* expression, whereas all WT seedlings were scored as rhythmic. These findings clearly demonstrate that CUL1 activity is important for establishment of the clock period and phase, as well as for maintenance of circadian rhythms under constant light.

Expression of core circadian clock genes in *axr6-3* mirrors that in *ztl* mutants

ZEITLUPE is a component of an SCF-type E3 ubiquitin ligase that plays a critical role in setting the circadian period (Mas *et al.*, 2003b; Somers *et al.*, 2004). Purification of the SCF^{ZTL} complex from plants yielded CUL1, which is consistent with these proteins participating in a common SCF complex (Han *et al.*, 2004). As full CUL1 activity is needed for correct setting of the circadian period and maintenance of accurate rhythms, the phenotype in *axr6-3* is potentially due to a reduction in ZTL activity, arising from a difficulty in assembling the SCF^{ZTL} complex. This possibility is supported by the observation that *axr6-3* seedlings capable of maintaining rhythms exhibit a long circadian period, similar to that seen in *ztl* mutants (Jarillo *et al.*, 2001; Somers *et al.*, 2000, 2004). Therefore, we assessed the phenotypic parallels between *axr6-3* and the loss-of-function mutant *ztl-4* by examining the levels of expression for the circadian clock genes *CCA1*, *LHY*, and *TOC1* in both mutant backgrounds.

ZEITLUPE is required in diurnal conditions for upregulation of the morning expression of *CCA1* and *LHY*, each encoding myb-like transcription factors that function at the core of the plant oscillator (Green and Tobin, 1999; Schaffer *et al.*, 1998; Somers *et al.*, 2004; Wang and Tobin, 1998). A phenotypic consequence of ZTL inactivation is a strong reduction in *CCA1* and *LHY* transcript levels. As expected, we found that *CCA1* and *LHY* levels in *ztl-4* were significantly reduced in seedlings grown under 12 h light:12 h dark cycles

Table 2 Estimates of free-running periods for *CAB2:LUC*, *CCR2:LUC*, and *TOC1:LUC* expression, and the proportion of seedlings exhibiting detectable rhythms in WT and *axr6-3* backgrounds

Genotype	<i>CAB2:LUC</i> ^a		<i>CCR2:LUC</i> ^a		<i>TOC1:LUC</i> ^a	
	Period \pm SD ^b (h)	Rhythmic/total	Period \pm SD ^b (h)	Rhythmic/total	Period \pm SD ^b (h)	Rhythmic/total
WT	24.2 \pm 0.6	25/26	23.6 \pm 0.6	18/18	24.0 \pm 0.5	26/26
<i>axr6-3</i>	25.6 \pm 0.8	23/27	25.0 \pm 0.7	24/25	27.2 \pm 1.4	19/29

Plants were entrained for 6 days under 12 h light:12 h dark cycles at 22°C, then held in continuous white light (50 μ mol m⁻² sec⁻¹) at 22°C for 5 days.

^aData are from two independent replicates.

^bThe variance-weighted mean period was determined as described previously (Millar *et al.*, 1995b; Plautz *et al.*, 1997). Only those period estimates with relative amplitude error values ≤ 0.6 and period estimates between 20 and 30 h were included in this analysis. SD, standard deviation.

at 22°C. The concentrations of *CCA1* and *LHY* mRNA were 4- and 10-fold lower, respectively, in *ztl-4* than in WT (Figure 3a,b). A comparable reduction in mRNA levels was observed in *axr6-3*: *LHY* and *CCA1* transcripts were down-regulated by approximately threefold in the *CUL1* mutant, relative to their levels in WT (Figure 3a,b). The diminished accumulation of *LHY* and *CCA1* transcripts in both mutant backgrounds was specific to these clock genes, as peak expression levels of *TOC1* in *axr6-3* and *ztl-4* remained close to those in WT (Figure 3c), as did expression levels of *CCR2* and *CAB2* (Figure S1a,b). Furthermore, the strength of the reduction in *CCA1* and *LHY* expression correlated well with the severity of the mutation in each background, as *axr6-3* showed a milder phenotype than the loss-of-function mutant *ztl-4*. In addition, both *ztl-4* and *axr6-3* showed a pronounced 4 h phase delay in the expression of *CCA1*, *LHY*, and *TOC1* (Figure 3a–c). The magnitude of this phase delay was similar to that observed in *axr6-3* for expression from promoter:*LUC* reporters under constant light (Figure 3). Thus, clock gene expression in *axr6-3* largely matched that observed in *ztl-4*, which suggests that the circadian phenotype of *axr6-3* resulted in large part from a reduction in ZTL activity.

Inhibition of CUL1 function in axr6-3 limits TOC1 degradation

In the late night, SCF^{ZTL} targets TOC1 for degradation by the ubiquitin/26S proteasome pathway (Mas *et al.*, 2003b). Circadian accumulation of TOC1 peaks at approximately 4 h after dusk, and the activity of ZTL is needed to impose this circadian rhythm on TOC1 levels (Mas *et al.*, 2003b). In the absence of ZTL, TOC1 levels are not rhythmic and the protein accumulates to high levels during the night.

We examined TOC1 stability in *axr6-3* to determine whether the circadian phenotype in *axr6-3* correlated with changes in TOC1 turnover, which would suggest an inhibition of SCF^{ZTL} activity in this mutant. The *axr6-3* allele was introduced into the well-studied *TOC1:TOC1-YFP* mini-gene (TMG) line, in which expression of a translational fusion of TOC1 and YFP is driven by the native TOC1 promoter (Mas

et al., 2003b). This line was used to assess the TOC1 expression waveform. In WT, TOC1-YFP exhibited the expected rhythmic expression profile at 22°C in 12 h light:12 h dark cycles; peak protein levels occurred in the evening at ZT14 and then declined with the approaching dawn (Figure 4a). Under the same conditions, TOC1 attained noticeably higher levels in *axr6-3* and exhibited an altered expression waveform (Figure 4a). The greatest TOC1 concentration differences between *axr6-3* and WT occurred between ZT22 and ZT2. In *axr6-3*, TOC1 did not exhibit the typical decline in concentration between ZT18 and ZT22 that marks the activity of SCF^{ZTL}; instead TOC1 levels fell only after the lights came on at ZT0. A trivial explanation for the modified TOC1 waveform in *axr6-3* TMG was that this new protein profile resulted from underlying changes in the expression pattern for the *TOC1-YFP* mRNA; however, *TOC1* mRNA was expressed in *axr6-3* TMG seedlings with a diurnal pattern comparable to that in the WT TMG background (Figure S2). In fact, *TOC1* expression levels were somewhat lower in the mutant than in WT. Thus, the altered TOC1 waveform in *axr6-3* was due to post-transcriptional changes in TOC1 accumulation and not to alterations in the abundance of *TOC1* mRNA. These data are consistent with the hypothesis that a defect in SCF^{ZTL} activity in *axr6-3* blocks TOC1 degradation during the night.

To confirm that the *axr6-3* allele affects the activity of the SCF^{ZTL} complex, the waveform of TOC1 accumulation under 12 h light:12 h dark cycles was determined at 18 and 28°C. These two conditions are expected to have opposite effects on TOC1 accumulation if *CUL1* is critical for SCF^{ZTL} activity. The TOC1 profile at 18°C was anticipated to more closely match that of WT, as this condition improves *axr6-3* leaf movement rhythms (Figure 1b). Conversely, we expected that TOC1 degradation in *axr6-3* would be reduced at 28°C, as this condition diminishes root growth inhibition by auxin and abolishes *CUL1* association with the SCF^{TIR1} complex (Quint *et al.*, 2005).

The TOC1-YFP waveform in *axr6-3* TMG seedlings grown continuously at 18°C was comparable to that observed for WT under the same conditions (Figure 4b). In contrast to the stable levels of TOC1 exhibited in *axr6-3* during the late night

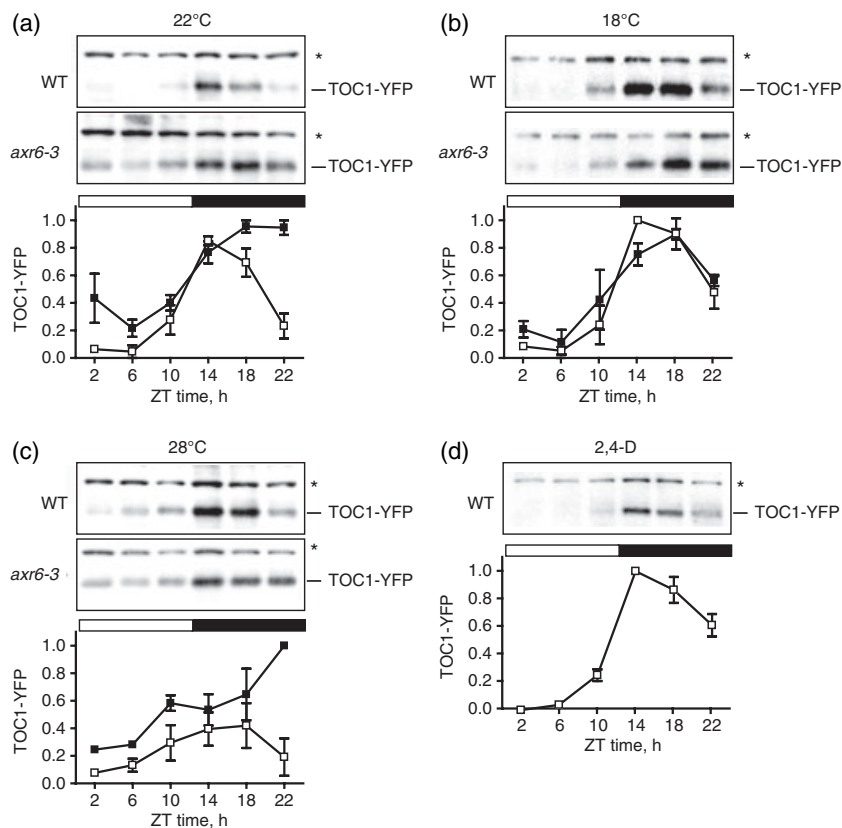


Figure 4. TOC1 turnover is altered in *axr6-3*, consistent with changes in SCF^{ZTL} activity.

(a–c) Normalized TOC1–YFP levels in WT and *axr6-3* *TOC1:TOC1*-YFP mini-gene seedlings grown under 12 h light:12 h dark conditions at 22°C (a), 18°C (b) or shifted from 22 to 28°C (c). (d) WT TMG seedlings grown at 22°C were sprayed at ZT0 with 0.3 μ M 2,4-D. Samples were taken at the indicated ZT times.

Upper panels, representative Western blots probed with anti-GFP. Lower panel, normalized average levels of TOC1–YFP in *axr6-3* (filled squares) and WT (open squares) from two independent replicates. Error bars represent the range. Asterisks indicate the nonspecific band used as a loading control and for normalization of TOC1–YFP levels. TOC1–YFP levels represent the ratio of the TOC1–YFP band intensity to that of the loading control. The TOC1–YFP level was then normalized to the highest value for each experiment.

at 22°C, a clear reduction in TOC1–YFP levels was apparent at the ZT22 time point in the mutant background, which is the time at which SCF^{ZTL} directs TOC1 to the 26S proteasome. Interestingly, peak TOC1 accumulation in *axr6-3* occurred 4 h later than in WT, which correlated well with the phase delay observed for *TOC1* expression in the mutant background (Figure S2). These results confirm that conditions permitting greater CUL1 activity in *axr6-3* resulted in a more substantial loss of TOC1–YFP at the time of night when SCF^{ZTL} normally promotes TOC1 turnover.

To test the effect of a temperature that considerably reduced CUL1 activity in *axr6-3*, mutant and WT TMG seedlings were grown under 12 h light:12 h dark cycles at 22°C and transferred to 28°C at ZT0 on the day of sample collection. The waveform of TOC1 accumulation in WT at this elevated temperature generally followed the same profile as observed at 22°C: maximal amounts of protein were present at ZT18 and then rapidly declined to trough levels by ZT2 (Figure 4c). In contrast, the expression profile of TOC1 in *axr6-3* was comparable to that observed in a *ztl* mutant, in which TOC1 degradation is inhibited (Mas *et al.*, 2003b). TOC1–YFP accumulated throughout the day to levels significantly higher than in WT. In addition, the concentration of the protein did not wane at ZT22, as it did in WT; instead, the amount of TOC1 continued to climb throughout the night. Clearly, temperature inactivation of CUL1 in the

axr6-3 background stabilizes TOC1 at the time when SCF^{ZTL} usually acts to remove this protein. Thus, ZTL appears unable to direct TOC1 for degradation in the absence of functional CUL1, consistent with CUL1 acting as the cullin protein in the assembly and activity of SCF^{ZTL}.

Exposure of seedlings to exogenous natural or artificial auxin moderately lengthens the free-running period and reduces the accuracy of rhythms (Covington and Harmer, 2007; Hanano *et al.*, 2006). For example, the presence of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) in growth medium at a concentration of either 0.3 or 5 μ M slows the pace of the clock by approximately 1 h and noticeably reduces the amplitude of rhythms (Covington and Harmer, 2007). Another synthetic auxin, 1-naphthaleneacetic acid (NAA), has similar effects on rhythms (Hanano *et al.*, 2006). Therefore, it is possible that the circadian phenotype in *axr6-3* might be explained by an auxin signaling defect instead of direct inhibition of SCF^{ZTL} activity. To examine this possibility, we determined the effect of 2,4-D application on the waveform of TOC1 accumulation. 2,4-D is a potent herbicide and its toxic effects might have indirectly changed TOC1 levels; therefore, we treated seedlings with 0.3 μ M 2,4-D, which is the lowest concentration shown to effectively lengthen the circadian period (Covington and Harmer, 2007). WT TMG seedlings sprayed with 0.3 μ M 2,4-D at ZT0 exhibited a

TOC1 accumulation profile in the 24 h following application of the hormone that matched the behavior of the protein in untreated seedlings (compare Figure 4a,d). As this acute 2,4-D treatment did not elicit a change in TOC1 degradation comparable to the effect in *axr6-3* at restrictive temperatures, defective auxin signaling appears unlikely to be a major contributor to the inhibition of TOC1 turnover that is evident in *axr6-3*.

Discussion

The temperature-sensitive *axr6-3* allele of *CUL1* was used to directly investigate the functional contribution of CUL1 to the plant circadian oscillator. We show that rhythmic leaf movements in *axr6-3* exhibit temperature-dependent arrhythmia. In addition, the period of circadian gene expression in *axr6-3* under restrictive temperature conditions is 1–3 h longer than in WT; furthermore, individual seedlings display substantial variability in period length and the robustness of their rhythms. Many aspects of the *axr6-3* phenotype correlated with those of a *ztl* mutant, including changes in the expression of core clock genes and a reduction in TOC1 degradation. Together, our results demonstrate that CUL1 is needed to set and maintain the circadian period, and that this is accomplished, at least in part, through its role in TOC1 turnover, which likely involves participation in the SCF^{ZTL} complex.

Circadian leaf movements in *axr6-3* were profoundly compromised, but less dramatic changes in circadian rhythms were apparent for *CCR2*, *CAB2*, and *TOC1* gene expression. A potential explanation for the difference between these two phenotypes is the fact that circadian leaf movement relies on multiple signaling pathways that each use CUL1. For example, petioles are the organs that support cotyledons in young Arabidopsis seedlings. Rhythmic leaf movements are produced by antiphase expansion of the abaxial (bottom) and adaxial (top) cells in the petioles (Engelmann and Johnsson, 1998). Auxin signaling contributes to regulation of cell expansion, and the auxin response is substantially blunted in *axr6-3* (Quint *et al.*, 2005). It follows that the auxin signaling defect in *axr6-3* is likely to limit the extent of petiole cell expansion, which, in turn, would render circadian rhythms difficult to detect because of weak overall leaf movements. Our data clearly demonstrate that circadian leaf movement in *axr6-3* is strongly compromised. For example, the circadian period for leaf movement varied substantially at the permissive temperature (18°C), and a greater proportion of seedlings were arrhythmic. These phenotypes were comparable to those seen for gene expression, indicating that leaf movement reflects important aspects of the *axr6-3* circadian phenotype.

The pace of the clock was slowed by *axr6-3*, and this change in period was observed for three circadian promoter:*LUC* reporter constructs; however, the severity of the

phenotype varied by reporter. The strongest gene expression phenotype was observed for *TOC1:LUC*. The period length for this reporter was approximately 3 h longer than for WT, which was two times greater than the change observed for *CAB2:LUC* and *CCR2:LUC*, which shared a 1.5 h slowing of the pace of the oscillator. In addition, the period length indicated by *TOC1:LUC* expression in the *axr6-3* population was distributed over a greater range than in WT, and a higher proportion of the seedlings exhibited poor rhythms. This same trend was seen when *TOC1:LUC* expression was compared to that of the other two bioluminescence reporters. Known repressors of TOC1 expression are the two myb-like transcription factors, CCA1 and LHY (Alabadi *et al.*, 2002; Matsushika *et al.*, 2002). In *axr6-3*, *CCA1/LHY* transcript levels were significantly reduced, but this did not result in an increase in *TOC1* expression. In fact, *TOC1* mRNA levels in the mutant were essentially the same as in WT. Therefore, the marked effect of *axr6-3* on the *TOC1:LUC* period cannot be explained by loss of LHY/CCA1-dependent control of *TOC1* expression.

Given that TOC1 is a core clock component, it seems counter-intuitive that the strong effects observed for *TOC1* expression were not directly translated into comparable changes in the rhythms exhibited by the bioluminescence reporters of the output genes *CAB2* and *CCR2*. A potential explanation for this situation is that CUL1 has separable contributions to the control mechanisms responsible for sculpting the TOC1 expression profile. The TOC1 waveform that ultimately contributes to clock parameters such as period length is shaped by transcriptional regulation at the *TOC1* promoter (Alabadi *et al.*, 2001; Matsushika *et al.*, 2002; Perales and Mas, 2007) and post-transcriptional control of TOC1 stability (Kim *et al.*, 2007; Mas *et al.*, 2003b). CUL1 appears to contribute to both these aspects of TOC1 regulation; the sensitivity of *TOC1* expression to the *axr6-3* allele suggests CUL1 activity may directly contribute to transcriptional control of *TOC1* expression, and functional CUL1 is clearly important for TOC1 degradation. As TOC1 protein is the active species needed to set the pace of output rhythms, the TOC1 degradation phenotype caused by *axr6-3* may mask the underlying transcriptional changes in *TOC1* expression. We propose that the expression of *CCR2* and *CAB2* reflect the ultimate effect of *axr6-3* on TOC1 accumulation, and that *TOC1:LUC* indicates the effects of diminished CUL1 activity on transcription of *TOC1*.

CUL1 activity appeared to be required to correctly set the circadian period and maintain robust rhythms under constant light conditions because *axr6-3* seedlings exhibited arrhythmic or long-period circadian gene expression. The molecular basis for this phenotype was most consistent with the limited TOC1 degradation observed in *axr6-3*. In the mutant, TOC1 accumulated to high levels at restrictive temperatures, and the severity of this phenotype was temperature-dependent. TOC1 turnover is known to be

mediated by the F-box protein ZTL, which suggests that the major effect of CUL1 inactivation on the clock was to impede SCF^{ZTL} activity. The RING protein RBX1 is a core component of SCF complexes, and is associated with SCF^{ZTL} *in vivo* (Han *et al.*, 2004). Transient silencing of RBX1 by RNA interference (RNAi) in Arabidopsis seedlings lengthens the circadian period for CCA1 accumulation (Han *et al.*, 2004). The RBX1 RNAi phenotype is analogous to the effect of *axr6-3* observed here for gene expression rhythms; thus, inhibition of SCF^{ZTL}, either by inactivation of RBX1 or CUL1, produces a long-period phenotype, similar to that of a *ztl* mutant. Our findings are consistent with the hypothesis that CUL1 contributes to TOC1 degradation by acting as the cullin constituent of SCF^{ZTL}, in agreement with previous work demonstrating that ZTL is physically associated with CUL1 *in vivo* (Han *et al.*, 2004).

Circadian rhythms in Arabidopsis are influenced by several phytohormones, including auxin (Covington and Harmer, 2007; Hanano *et al.*, 2006). Exposure of seedlings to exogenous natural auxin indoleacetic acid (IAA) or the artificial auxins NAA, and 2,4-D lengthens the free-running period by an average of 1 h for bioluminescence rhythms exhibited by six promoter:LUC reporters constructs, including *CCR2:LUC*, *CAB2:LUC*, and *TOC1:LUC* (Covington and Harmer, 2007; Hanano *et al.*, 2006). Also, Arabidopsis seedlings grown on medium containing 2,4-D exhibit a marked decrease in the amplitude of bioluminescence rhythms (Covington and Harmer, 2007), which notably includes the expression of *CCA1* and *TOC1*. NAA has similar effects on the amplitude of expression of *CCA1*, *CCR2*, and *CAB2*, and this auxin also causes reduced clock precision (Hanano *et al.*, 2006).

The pharmacological effects of auxin application on circadian rhythms are reminiscent of the clock phenotypes observed here for *axr6-3*. Given that *axr6-3* is an auxin signaling mutant, the circadian phenotype in this background could be potentially explained by auxin signaling defects that feed back to influence the circadian oscillator. We do not favor this possibility because of the molecular phenotype exhibited by *axr6-3*. We found that acute treatment of WT seedlings with a concentration of 2,4-D known to slow the pace of the oscillator did not produce an inhibition of TOC1 degradation comparable to that observed in *axr6-3* at either of the restrictive temperatures tested here. Furthermore, the fact that *axr6-3* exhibits a marked phase delay for both gene expression and TOC1-YFP accumulation is inconsistent with an auxin signaling defect, as this phytohormone does not contribute to setting of the clock phase or promote phase shifting of circadian rhythms (Covington and Harmer, 2007). Although a contribution of auxin signaling to the *axr6-3* phenotype remains a formal possibility, the data presented here are most consistent with a reduction in TOC1 degradation arising from a temperature-dependent inhibition of CUL1 that affects ZTL activity.

If the major circadian defect in *axr6-3* stems from reduced turnover of TOC1, then is it possible that this is the only regulatory point where CUL1 participates in circadian signaling? We think that is unlikely. Three explanations can be advanced to account for the predominance of the *ztl*-like phenotype in *axr6-3*. First, sufficient CUL1 activity may exist in *axr6-3* such that other clock-associated SCF complexes can still assemble and bind to their cognate targets, either because the targets for these SCF complexes are expressed at relatively low levels, or because the associated F-box protein is highly expressed. On the other hand, effective TOC1 turnover may require nearly full SCF^{ZTL} activity, because TOC1 and SCF^{ZTL} exist at stoichiometric levels in the cell. If this were the case, then TOC1 levels would be tightly coupled to the absolute amount of SCF^{ZTL} activity, and, as a result, TOC1 accumulation would respond proportionally to changes in activity of the E3 ligase. As circadian period is responsive to the balance between the activities of TOC1 and ZTL, this system would act as a molecular rheostat that finely controls clock period in response to changes in either ZTL activity or TOC1 expression.

Second, one or more of the alternative CUL proteins in Arabidopsis may substitute for CUL1 in other clock-associated SCF complexes. In support of this possibility, ethylene and GA responses are not dramatically affected in *axr6-3*, even though EBF1/2 and SLY1 associate with CUL1 *in vitro* (Potuschak *et al.*, 2003; Xu *et al.*, 2002). Possibly, the SCF^{EBF1/2} and SCF^{SLY1} complexes are capable of incorporating a cullin other than CUL1, and thereby maintain a proper response to these hormones in *axr6-3*. Finally, the phenotype produced by inhibition of TOC1 degradation may be sufficiently strong to mask any other phenotypic effects resulting from CUL1 inactivation.

In conclusion, our results demonstrate that CUL1 is required for TOC1 degradation, most likely by affecting SCF^{ZTL} activity, and therefore CUL1 functions as part of the system responsible for defining clock period and ensuring precise rhythms. These findings show the importance of selective protein turnover mediated by the ubiquitin/26S proteasome pathway in the molecular mechanism of the plant circadian clock.

Experimental procedures

Plant materials and growth conditions

All plants were in the Columbia background. *ztl-4* corresponds to the T-DNA line SALK_35701 (Alonso *et al.*, 2003); the insert is present at base pair 2216, which interrupts the portion of the gene encoding the second kelch repeat (Figure S3a). ZTL transcript expression was diminished fourfold in this background (Figure S3b). The period for *CAB2:LUC* expression in *ztl-4* was 27.45 ± 0.38 h compared with 24.19 ± 0.45 h in WT (Figure S3c). Surface-sterilized seeds were plated on 1× MS medium (Murashige

and Skoog, 1962; Caisson Laboratories, <http://www.caissonlabs.com>) with 0.8% agar and 3% sucrose, stratified in the dark at 4°C for 3–5 days, and then transferred to the indicated conditions. Light was provided by cool white fluorescent bulbs, at an intensity of approximately 50 $\mu\text{mol m}^{-2} \text{sec}^{-1}$.

Leaf movement

Seedlings for experiments determining leaf movement were grown under 12 h light:12 h dark cycles for 5 days at the indicated temperature. On day 6, seedlings were transferred to continuous white light (approximately 50 $\mu\text{mol m}^{-2} \text{sec}^{-1}$) for 5 days. Video-recorded images of seedlings under constant light were acquired every 20 min. Curve-fit analysis was used to determine estimated period lengths from the cotyledon position information (Plautz *et al.*, 1997). The data for each seedling were analyzed by the Fast Fourier Transform/Nonlinear Least Squares method (FFT/NLLS), which fits the data to a modified cosine curve, to obtain estimated period length and relative amplitude error. Relative amplitude error is a statistical measure of how well the data conform to an ideal cosine curve, where a perfect match corresponds to a value of 0 and no correlation is indicated by a value of 1 (Millar *et al.*, 1995a). A cut-off of ≤ 0.25 for the relative amplitude error was used to identify robust rhythms in this data.

Bioluminescence assay

axr6-3 was introduced into *CCR2:LUC* (Alabadi *et al.*, 2001), *CA-B2:LUC* (Millar *et al.*, 1992), and *TOC1:LUC* (Alabadi *et al.*, 2001) reporter lines by crossing. Seedlings harboring the reporter construct were grown for 6 days under 12 h light:12 h dark cycles at the indicated temperature. On the morning of the seventh day, seedlings were transferred to continuous light conditions. Imaging of bioluminescence rhythms was performed as described previously (Millar *et al.*, 1992; Somers *et al.*, 1998). Estimated period length was determined by FFT/NLLS analysis. A cut-off of ≤ 0.6 for the relative amplitude error was used to identify robust rhythms in this data.

Whole-cell extracts and Western blots

Whole 10–14-day-old seedlings grown under the indicated conditions were harvested at the indicated times and flash frozen in liquid nitrogen. For experiments at 28°C, seedlings were transferred to 28°C at ZT0 (08:00 h) on the day of sample collection. Auxin treatments were applied at ZT0 and consisted of spraying approximately 100 seedlings on a 90 cm² square Petri plate with 1.5 ml of 0.3 μM 2,4-D (Sigma, <http://www.sigmaaldrich.com/>) diluted in 0.1% Triton X-100. The 2,4-D solution used was prepared from a 20 μM stock in ethanol. Tissue was disrupted by grinding in a mortar and pestle cooled with liquid nitrogen. Whole-cell extracts were prepared by vortexing ground tissue with an equal volume of extraction buffer (50 mM HEPES/NaOH, pH 7.5, 100 mM NaCl, 10% glycerol, 5 mM EDTA, 5 mM DTT, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 5 mM benzimidazole, EDTA-free protease inhibitor (Roche, <http://www.roche.com>), 1 mM PMSF (phenylmethanesulfonyl fluoride), 50 μM MG132 (Peptides International, <http://www.pepnet.com>) and 1% polyvinylpolypyrrolidone). The lysate was cleared by centrifugation at 17 000 g for 30 min at 4°C. Total protein concentration was determined using the reducing agent-compatible BCA protein assay (Pierce, <http://www.piercenet.com>). Equal amounts of total protein (40–80 μg) were separated on 6% SDS-PAGE gels as described

previously (Sambrook and Russell, 2001). Proteins were transferred to a 0.2- μm nitrocellulose membrane, and the TOC1-YFP fusion was detected using rabbit anti-GFP horseradish peroxidase (HRP) conjugate (Abcam, <http://www.abcam.com>). HRP activity was determined using SuperSignal West Pico chemiluminescent substrate (Pierce) and BioMax film (Kodak, <http://www.kodak.com>). TOC1-YFP levels were determined as the ratio of the integrated density of the TOC1-YFP band to that of a nonspecific band serving as the loading control (indicated by an asterisk in Figure 4). The integrated density of the protein bands was measured using IMAGEJ software (Abramoff *et al.*, 2004) from scanned tiff images of the films from each blot.

Expression analysis by real-time PCR

Seedlings were grown, harvested and ground as described above for immunoblot analyses. Total RNA was isolated using the Plant RNeasy Mini Kit (Qiagen, <http://www1.qiagen.com>). First-strand cDNA was generated from 1 μg of total RNA using the SuperScript II first-strand synthesis for RT-PCR kit (Invitrogen, <http://www.invitrogen.com>). This cDNA was diluted 1:4 to produce a working stock, and 2 μl of the working stock was used as template for PCR amplification using a iCycler iQ Real-Time PCR Detection System (Bio-Rad, <http://www.bio-rad.com>), to determine the expression of *TOC1*, *LHY*, and *CCA1* using the conditions, primers and probes described previously (Hazen *et al.*, 2005). Expression of *IPP2* (isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, At3g02780) was used as a normalization control, and its expression was determined using the primers and probes described previously (Hazen *et al.*, 2005). The expression level for each gene was calculated using the formula $2^{[C_T(IPP2) - C_T(\text{experimental})]}$, where C_T is the average threshold cycle for three technical replicates. *ZTL* expression was determined using 5'-TCAGCGTCTCAGCTTTATCTAC-3' as the forward primer and 5'-GAGGCGGTCTTCTGGAATG-3' as the reverse primer. For this primer set and the corresponding *IPP2* controls, PCR conditions were 95°C for 1 min, followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C in a buffer consisting of 1 \times ExTaq buffer (Takara Bio USA, <http://www.takarabiousa.com>), 1 \times EvaGreen (Biotium, <http://www.biotium.com>), 0.1% v/v Tween-20, 5% v/v DMSO, 50 $\mu\text{g ml}^{-1}$ BSA (New England Biolabs, <http://www.neb.com>), 0.2 mM dTNPs, 0.3 μM primers and 0.5 U Taq polymerase (New England Biolabs).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Figure S1. Expression of *CAB2* and *CCR2* under light:dark cycles.

Figure S2. *TOC1* expression in *axr6-3* and WT TMG lines.

Figure S3. Characterization of *ztl-4*.

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